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Isolation and evaluation of antimicrobial activity against *Staphylococcus* spp strains isolates from bovine mastitis in Ecuador

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ABSTRACT

Background and Objective: Bovine mastitis, particularly caused by *Staphylococcus* spp., poses challenges in dairy production. This study assessed the antimicrobial activity of various antibiotics on strains isolated in Ecuador. **Materials and Methods:** Milk samples from cows with mastitis were collected in Ecuador, isolating and identifying *Staphylococcus* spp. The strains were tested against antibiotics using the disk diffusion method. **Results**: Streptomycin exhibited the highest effect, with an average halo diameter of 27.86 mm (30µg), followed by neomycin with 25.40 mm (30µg) and gentamicin with 24.80 mm (30µg). However, high resistance to beta-lactams was observed, with penicillin reaching only 23.26 mm (30µg). These findings suggest inefficacy in current therapeutic treatments, especially in mastitic cases treated with the analyzed drugs. It is crucial to consider these findings to adapt therapeutic strategies addressing bacterial resistance and improving treatment efficacy in dairy cattle. **Conclusion**: Continuous monitoring of antimicrobial sensitivity is essential for optimizing livestock health and productivity, adjusting treatment protocols according to bacterial resistance evolution.

Keywords: *Staphylococus aureus*, *Staphylococus* spp, bovine mastitis, antimicrobial activity.

INTRODUCTION

Bovine mastitis is an inflammatory disease of the mammary glands of cows, which can be caused by different infectious agents, with bacteria being the most common.

This condition represents one of the greatest challenges for the dairy industry worldwide, as it significantly affects milk production and quality, in addition to generating high economic costs due to treatments and associated losses[1].

This disease can present clinically, where visible signs such as swelling, redness, and pain in the udders, as well as changes in the texture and color of the affected milk, are observed, or subclinically, where no evident symptoms are manifested, but there is still the presence of bacteria in the milk and a decrease in milk production[2].

Mastitis can be caused by various factors, including inadequate management conditions, stress, udder traumas, environmental contamination, among others. Hygiene and proper milking parlor management are crucial to prevent disease spread, as well as the prudent use of antibiotics for treating diagnosed cases[3].

Bovine mastitis does not directly cause illnesses in consumers through the consumption of contaminated milk. However, the presence of pathogenic bacteria in the milk from cows affected by mastitis can pose a risk to human health if proper pasteurization protocols and hygienic handling of dairy products are not followed[4].

The bacteria that cause bovine mastitis, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, and others, can produce toxins or contaminate milk with pathogens that could cause illnesses in consumers if ingested in sufficient quantities[5].

Therefore, it is essential to ensure that raw milk undergoes an appropriate pasteurization process before consumption, as this thermal process eliminates the pathogenic bacteria present in the milk, thus reducing the risk of foodborne illnesses. Prevention and control of mastitis are essential for maintaining herd health and the profitability of dairy production [6].

The genus *Staphylococcus* is a group of Gram-positive, spherical bacteria, clustered in grape-like arrangements. They are ubiquitous microorganisms found on the skin and mucous membranes of humans and animals. *Staphylococcus* is divided into different species, some of which are pathogenic to humans and animals, while others are commensal or even beneficial[7].

In the context of bovine mastitis, *Staphylococcus aureus* is one of the most common causes of this disease. This bacterium can enter the cow's udder during the milking process, especially if proper hygiene practices are not maintained. Once in the udder, it

can cause inflammation, infection, and production of toxins that affect milk quality and cow health[3].

Mastitis caused by *Staphylococcus aureus* can be clinical or subclinical. In the clinical form, evident signs of inflammation in the udder are observed, such as redness, swelling, and presence of pus in the milk. In the subclinical form, no external signs are observed, but milk production may decrease and elevated somatic cells may be found in the milk[8]. With this analysis, the isolation of *Staphylococcus aureus* in bovine mastitis is crucial for accurate diagnosis, appropriate treatment selection, and epidemiological control. It allows for strain identification, understanding antibiotic resistance, and developing prevention strategies. Furthermore, in dairy production, its detection ensures food quality and safety, indicating possible deficiencies in hygiene[9]. The characterization of *Staphylococcus aureus* involves a series of biochemical tests, such as catalase, coagulase, carbohydrate fermentation (such as mannitol), production of lactic acid, and other bacterial identification tests. These tests provide details about its metabolism and pathogenic potential, crucial for establishing its phenotypic profile, understanding its physiological characteristics, and differentiating it from other bacterial species[10],[11].

Bacterial resistance to antibiotics is a serious public health issue. *Staphylococcus*, especially *Staphylococcus aureus*, has developed resistance to multiple antibiotics, including beta-lactams such as methicillin (MRSA), and other common types of antibiotics[12]. This is mainly due to natural selection induced by the excessive and inadequate use of antibiotics, as well as the transfer of resistance genes between bacteria. *Staphylococcus* resistance to antibiotics can significantly complicate the treatment of infections, increase morbidity and mortality, and prolong the duration of the disease[12]. It is essential to adopt strategies for rational antibiotic use and promote infection control measures to address this emerging public health problem. Conducting antimicrobial activity analyses is crucial to detect pathogen resistance, such as *Staphylococcus*, to antibiotics. These analyses allow identification of which antibiotics are effective against bacterial strains and which are not, guiding appropriate infection treatment[13]

With all this background, the objective of the present study was to evaluate the sensitivity of *Staphylococcus* spp. strains isolated from cases of bovine mastitis in Ecuador to antibiotics from the beta-lactam, cephalosporin, aminoglycoside, and tetracycline families, to determine resistance patterns and guide appropriate treatment strategies.

MATERIALS AND METHODS

This research was conducted in the general laboratory of the Faculty of Agricultural Sciences, Natural Resources, and Environment of the State University of Bolívar, Ecuador. The samples were collected from 136 cows from 7 farms of the Agricultural Cooperative "La Colina," located in the San Pedro area, San Miguel de Los Bancos, province of Pichincha, Ecuador.

Detection of mastitis in milk through CMT (California Mastitis Test) in the field.

A physical examination of the animals was conducted before milking to identify signs of mastitis. Then, the CMT (California Mastitis Test) was applied in a ratio of 2:1 (milk: reagent) according to Balemi et al.[14] and the protocol by Maldonado et al.[15].

Establishment of the mastitis prevalence rate in the study area

The prevalence was determined by recording mastitis-positive animals, for which the formula described by Alvarado *et al.* [16].

$$\%\ \textbf{Prevalence} = \frac{Number\ of\ cases\ affected\ by\ the\ disease}{\text{Total number of the population}}\ x\ 100$$

Isolation by plate culture.

After detecting a cow with clinical or subclinical mastitis and that has not received antibiotic treatment, a sample from the affected quarter was collected. The teat was disinfected with 70% alcohol, teat end was trimmed and discarded, and the milk sample was collected in a labeled and coded test tube. The sample was stored in a refrigerated cooler at 4-8°C.

The sample was enriched with peptone water and packed into screw-capped tubes at a ratio of 9:1 (peptone water:milk) (Acumedia, Lot. 107596ª, USA). After cooling to 40°C, it was incubated for 24 hours at 37°C. Then, it was plated on blood agar base (Difco, Lot.0237250, USA) with 5% sheep blood to isolate Gram-positive bacteria.

After the initial incubation, the culture was duplicated and triplicated to purify the bacterial strain through triple streaking. Following incubation for 24 hours at 37°C, growth of a single bacterial type was obtained. The resulting bacterial colonies underwent

biochemical tests to identify their specific metabolic pathway, allowing for precise identification.

Identification and characterization of the bacteria

Phenotipic identification

In the bacterial identification process, metabolic pathways for sugar nutrition and fermentation were considered, along with colorimetric indicators in specific and differential media. To identify Gram-positive bacteria like *Staphylococcus aureus*, Mannitol Salt Agar (Oxoid, REF: CM0085, LOT: 3289843, UK) and Tryptic Soy Agar (Difco, Lot.1153604, USA) were used, allowing selective growth of this bacterial genus. Additionally, morphological identification at the cellular level was performed using Gram staining. These techniques were crucial for discerning the presence of the pathogenic bacteria.

Biochemical tests for bacterial characterization

For the Methyl Red test, the reagent was diluted in 96% methanol and 5 drops were applied to a tube with a bacterial suspension. A red coloration of the medium indicates mixed acid fermentation, considered positive. In the Oxidase test, the appearance of dark purple color after inoculation indicates a positive result. In Catalase test, bubble formation upon addition of hydrogen peroxide indicates a positive result. These tests, crucial in bacterial identification, allow discernment between positive and negative results based on specific reactions[17],[18].

The coagulase test was also performed, for which rabbit blood was collected in Vacutainer tubes with lavender caps containing anticoagulant (EDTA), centrifuged for 5 minutes at 3000 rpm, and plasma was collected. 0.5 mL of plasma was transferred to Eppendorf tubes and bacteria were suspended. After 4-6 hours of incubation at 37 °C, clot formation at the bottom of the tube was observed, indicating a positive result. Absence of clotting was considered negative[19],[20],[21]. The biochemical characterization profile is shown in Table 1.

Table 1. Biochemical identification profiles for Gram-positive bacteria isolated from bovine mastitis.

Test / **Isolates**	Coagulase (+/-)	Catalase (+/-)	Oxidase (+/-)	Mannitol fermentation.	Hemolysis Pattern
S. aureus.	+	+	-	+	Beta
Staphyloccocus spp	-	+	+	V	V

Assessment of the antimicrobial activity of the investigated drugs against the target pathogen.

The antimicrobial activity was conducted using the Kirby-Bauer method, antibiotic disk diffusion at concentrations proposed in the research (1µg, 5µg, 10µg, and 30µg). Plates were inoculated using the McFarlan 0.5 scale (USGS, USA), then incubated for 24 hours at 37°C. Subsequently, the bacterial growth inhibition zone was measured, establishing susceptibility antibiotics by CLSI[22].

A bacterial suspension of 1.5 CFU/mL was prepared in sterile distilled water, and its turbidity was adjusted using the McFarland 0.5 scale to ensure an appropriate concentration. Subsequently, the culture medium was inoculated with a sterile swab in four directions for even distribution of the microorganism. Filter paper disks with antibiotics at different concentrations were then placed on the medium's surface, maintaining adequate separation. Finally, the Petri dish was sealed with parafilm, and a unique code was recorded to prevent contamination and ensure traceability. After 18-24 hours of incubation, the formation of inhibition zones around the disks was evaluated[23].

The antibiotics analyzed against *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. were: Oxytetracycline (5µg, 10µg, and 30µg); Beta-lactams: Penicillin, Cephalexin (5µg, 10µg, and 30µg), also Cloxacillin (1µg, 5µg, 10µg); Aminoglycosides: Gentamicin, Neomycin, and Streptomycin (5µg, 10µg, and 30µg).

Estimation of the minimum inhibitory concentration (MIC) of the drugs under study.

The MIC, minimum inhibitory concentration, is determined (in µg/ml) using the Kirby-Bauer method. The diameter of the zones formed around the disks is measured with a vernier caliper. Susceptibility criteria are based on CLSI, VET01S and guidelines[24],[25]

RESULTS AND DISCUSSION

Determination of mastitis prevalence on the farms.

According to the CMT results, the mastitis prevalence was 36.76% out of a total of 136 animals, with 50 positive cases. Farm 6 showed the highest prevalence (45%), while Farm 1 had the lowest (18.2%). These figures align with previous research by Avellan et al. [26] and Sánchez-Bonilla[27], indicating that mastitis is present when udder milking and sanitation protocols are inadequate.

Isolation and identification of the pathogens that cause mastitis.

Fifty-one bacterial strains were isolated and identified from mastitis-positive samples of 50 analyzed cows. According to biochemical analysis, *Staphylococcus aureus* had the highest prevalence (29.42%, 15 isolates), followed by coagulase-negative Staphylococcus spp. (15.68%, 8 isolates). In comparison with other studies, pathogen distribution varies according to geographical location and sanitary management practices during milking[28].

Antimicrobial activity of Oxytetracycline against *Staphylococcus aureus*

Table 2. Analysis of antimicrobial susceptibility of Oxytetracycline against *S. aureus* at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Oxytetracycline	5.13 mm	100 %	8.53 mm	100 %	17.66 **mm**	**6.7** %

The results showed total resistance in all isolates to 5µg and 10µg of antibiotic, while in the 30µg, resistance was 6.7%, with a larger halo size. According to Arroba[29], in mastitis milk samples, S. aureus exhibited 91.6% resistance to tetracycline. Lucas et al[30]. reported a 59% resistance in *S. aureus* to tetracyclines. This suggests variability in oxytetracycline resistance, influenced by various factors.

Antimicrobial activity of Beta-lactams (Penicillin, Cephalexin and Cloxacillin) against *Staphylococcus aureus*.

Table 3. Antimicrobial susceptibility analysis of Beta-lactams against *S. aureus* at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Penicillin	5.66 mm	100 %	11.4 mm	100 %	**23.26 mm**	**80** %
Cephalexin	3.40 mm	100 %	7.4 mm	100 %	**17.13 mm**	**80** %
Cloxacillin	**1µg**		**5µg**		**10 µg**	

	3.66 mm	100 %	10.06 mm	100 %	**20.20 mm**	**80** %

The most effective treatment proved to be the administration of 30 µg of Penicillin, followed by 10 µg of Cloxacillin and 30 µg of Cephalexin. Intermediate treatments, such as 10 µg of Penicillin, 5 µg of Cloxacillin, and 10 µg of Cephalexin, showed no significant differences among themselves, respectively. In contrast, treatments with the lowest averages were 5 µg of Penicillin, 1 µg of Cloxacillin, and 5 µg of Cephalexin (see Table 3).

In the case of Penicillin, both the treatments with 5 µg and 10 µg showed resistance in all isolates, while the treatment with 30 µg exhibited a resistance of 80%. Previous findings, such as those of Srednik et al. [31], corroborate similar results regarding resistance to penicillin.

Regarding Cephalexin, the treatment with 30 µg revealed intermediate resistance of 80%, while treatments with 1 µg and 5 µg of Cloxacillin showed resistance in all isolates. Previous research, such as that of Neder et al.[32], indicates that Cephalexin had a sensitivity of 99% and only a resistance of 1.04%.

Finally, the treatment with 10 µg of Cloxacillin showed a resistance of 80%. Previous studies, such as Abdul et al. [33], found a sensitivity of 72.42% to 10 µg of Cloxacillin.
The data suggest that resistance is linked to the agroecological zone, mastitis treatment management, indiscriminate antibiotic use without clinical criteria, and differences in resistance due to environmental factors and improper antimicrobial management.

Antimicrobial activity of Aminoglycosides (Gentamicin, Neomycin and Streptomycin) against *Staphylococcus aureus*.

Table 4. Antimicrobial susceptibility analysis of Aminoglycosides against *S. aureus* at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Gentamicin	6 mm	100 %	17.33 mm	13 %	24.80 **mm**	**100** %
Neomycin	6.13 mm	100 %	13.20 mm	33.3 %	25.40 **mm**	6.7 %
Streptomycin	5.66 mm	100 %	11.93 mm	46.67 %	27.86 **mm**	**0** %

When comparing antibiotics at 30 µg, Streptomycin showed the highest average in mm of halo, followed by Neomycin and Gentamicin. At the concentration of 10 µg,

Gentamicin had the highest average, while Neomycin and Streptomycin had similar ranges with no significant differences. In the 5µg treatments, the averages showed no significant differences and were the smallest halo sizes.

In the analyzed context, it was observed that with 5 µg of Gentamicin, all isolates showed resistance, while with 10 µg, only 2 isolates were resistant. At a concentration of 30 µg, a sensitivity of 100% was evidenced. According to Abdul et al. [33], the research indicates a sensitivity of 93.08% to 10µg of Gentamicin, suggesting its ability to inhibit growth under similar conditions.

When analyzing Neomycin, it was observed that at 5 µg, all isolates showed resistance, while at 10 µg, 33.33% were resistant. At 30 µg, only 6.7% of the isolates showed resistance. In a previous study, Abdul et al. [33], found a sensitivity of 87.93% for Neomycin (30µg), suggesting adequate efficacy of the drug.

Regarding Streptomycin, the findings indicate that at 5 µg, all isolates show resistance, while at 10 µg, resistance is 46.67%. However, at 30 µg, a sensitivity of 100% is observed.

Antimicrobial activity of oxytetracycline against coagulase-negative *Staphylococcus* spp

Table 5. Antimicrobial susceptibility analysis of oxytetracycline against coagulase-negative *Staphylococcus* spp at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Oxytetracycline	11.25 mm	100%	22.50 mm	50%	35.87 mm	**0**

After analysis, it was determined that the most effective treatment was at 30µg, followed by 10µg and 5µg, with statistically significant differences. Regarding resistance, the treatment with 5µg showed resistance in all isolates, while the 10µg treatment revealed that half were resistant (37.5% intermediate resistance and 12.5% total resistance).

On the other hand, the treatment with 30µg demonstrated 100% susceptibility in the 8 isolated bacteria. These results are consistent with previous studies by Arroba[29] and Sánchez et al. [34] regarding susceptibility to tetracyclines, supporting the effectiveness of certain concentrations according to bacterial sensitivity.

Antimicrobial activity of beta-lactams (Penicillin, Cephalexin, and Cloxacillin) against coagulase-negative *Staphylococcus* spp.

Table 6. Antimicrobial susceptibility analysis of beta-lactams (Penicillin, Cephalexin and Cloxacillin) on *Staphylococcus* spp CN at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Penicillin	6.62 mm	100 %	13.87 mm	100 %	26.12 **mm**	**100** %
Cephalexin	5.62 mm	100 %	9.87 mm	100 %	21.25 **mm**	%
Cloxacillin	**1µg**		**5µg**		**10 µg**	
	4.87 mm	100 %	9 mm	100 %	18.87 **mm**	%

At higher concentrations, 30µg of Penicillin had the best average, followed by Cephalexin and 10µg of Cloxacillin. At 10µg, Penicillin was equally more effective, followed by Cephalexin and 5µg of Cloxacillin. At 5µg, Penicillin showed the best result, followed by Cephalexin and 1µg of Cloxacillin.

In the resistance analysis, the 8 isolates showed resistance to all three antibiotic concentrations. However, in the study by Sánchez et al. [27], 27, 58% resistance and 42% sensitivity to penicillin were identified, contrasting with the 100% resistance observed in the coagulase-negative *Staphylococcus* spp. isolates in this study.

On the other hand, all isolates showed resistance to all three concentrations of Cephalexin. Arroba[29] reported 97% sensitivity and 3% resistance in their study of 33 strains of Staphylococcus spp. to Cephalexin (30µg), in contrast to the total resistance observed in this study, likely due to improper antibiotic management.

Regarding Cloxacillin, the results indicate that all isolates were resistant to all three concentrations of the antibiotic. According to Cruz et al. [35], in their study of 15 coagulase-negative Staphylococcus spp. strains subjected to Cloxacillin (5µg), 27.2% resistance was observed, contrasting with the 100% resistance in the current isolates.

Antimicrobial activity of Aminoglycosides (Gentamicin, Neomycin, and Streptomycin) against coagulase-negative *Staphylococcus* spp.

Table 7. Analysis of antimicrobial susceptibility of Aminoglycosides (Gentamicin, Neomycin, and Streptomycin) against coagulase-negative *Staphylococcus* spp. at three concentrations

Antibiotic	**Concentrations / Resistance**					
	5µg		**10 µg**		**30µg**	
Gentamicin	15.75 mm	0 %	27.75 mm	0 %	51.75 **mm**	**0** %

Neomycin	16.12 mm	0 %	30.37 mm	0 %	54.87 **mm**	0 %
Streptomycin	18.75mm	0 %	33.12 mm	0 %	53.87 **mm**	**0** %

El análisis revealed that Neomycin 30µg and Streptomycin 30µg were the most effective treatments. These treatments did not show significant differences between each other. At a concentration of 10µg, Streptomycin stood out, closely followed by Neomycin. At concentrations of 5µg, Streptomycin led with an inhibition zone exceeding 18.75 mm (Table 7).

The results with Gentamicin indicate that the 8 isolates are susceptible to the three concentrations studied. In contrast, according to Arroba[29], Gentamicin (10µg) exhibited an 82.6% resistance in 46 isolates. Similarly, Neomycin inhibited the growth of 100% of the isolates. However, Arroba[29] found a resistance level of 95.6% at 30µg, which contrasts with our findings.

The same effect was evidenced with the antibiotic Streptomycin at the three concentrations studied, according to Sánchez et al. [27], 77.41% of the strains are sensitive and 22.59% are resistant to Streptomycin at 10 µg.

Conclusion

The antimicrobial sensitivity of coagulase-negative Staphylococcus spp. isolates causing bovine mastitis varied significantly depending on the concentration and type of antibiotic used. High sensitivity to certain antibiotic concentrations was observed, such as in the case of Streptomycin at 5µg, where 100% of the isolates were sensitive, suggesting the need to consider specific doses and agents in the treatment of this disease.

The results obtained differ somewhat from previous studies, suggesting a possible evolution in antimicrobial resistance of coagulase-negative Staphylococcus spp. over time. This underscores the importance of continuously monitoring the effectiveness of antibiotics and adapting treatment strategies according to changing resistance trends in bacterial populations.

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